Research article

IκBNS and IL-6 expression is differentially established in the uterus of pregnant healthy and infected mice

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ABSTRACT

During pregnancy, NF-κB plays an important role for embryo implantation and the onset of labor. Regulated IL-6 production, under transcriptional control of NF-κB, is essential for a successful pregnancy outcome and the atypical regulator IκBNS is involved in this process. Previously, we showed that IκBNS negatively regulates IL-6 in uterine tissues during mouse estrous cycle. In this work, we analyzed if IκBNS and IL-6 expression in pregnant mice under physiological or *L. monocytogenes*-infected conditions would remain as observed in estrous cycle. In the healthy pregnancy IL-6 was highly expressed during implantation/placentation and labor stages but decreased during fetal development and *post-partum* stages. In contrast, in mice infected before pregnancy, IL-6 expression was not increased in the implantation stage, and its regulator IκBNS increased more in the infected condition rather than in the healthy pregnancy. IκBNS expression was reduced in post-implantation infection, allowing for IL-6 overexpression. The IκBNS-unrelated cytokine IL-36γ, used as inflammatory cytokine marker, was severely increased in the infected uterine tissues. When we analyzed the effect of infection over the fetuses, we found that pre-implantation infection caused the resorption (rejection) of some products, while the post-implantation infection restricted the intrauterine growth of fetuses. The results suggest that in the uterine tissue of pregnant mice the regulatory effect of IκBNS over IL-6 is more evident in an infection status rather than in a healthy condition.

1. Introduction

According to global statistics, one out of ten childbirths is preterm, turning this condition into the leading cause of infant morbidity and mortality [1, 2]. In Mexico, the average rate of preterm labor (PTL) is similar to developed countries; however, this percentage can be as high as 40% in the poorest regions [3]. Bacterial intrauterine infections, marked by microbial invasion of amniotic fluid (MIAC) and/or premature rupture of membranes, are the most common cause of PTL, representing at least 40% of the total cases [4]. Watts *et al.* indicate that the

earlier the presence of PTL in gestational age, the higher the rate of MIAC [5]. Lack in the identification of molecular mechanisms that limit and regulate the trigger of delivery, has segregated diagnosis, prevention and treatment of preterm labor as a topic of interest in public health. NF-κB is a key molecule modulating not only labor, but also implantation stage in pregnancy [6, 7, 8, 9], since the pro-inflammatory cytokines (IL-6 included) required for these processes are transcriptionally regulated by NF-κB and are also found up-regulated in normal and PTL in fetal-maternal membranes [10, 11, 12], placenta [7], and myometrium [13, 14, 15, 16]. IκBNS belongs to the family of atypical regulators of

NF-κB (IκBs), which have a nuclear localization and are generally not expressed in unstimulated cells but can be induced after cell activation with several stimuli [17]. IκBNS plays a role in thymocytes undergoing negative selection [18], is important for TLR-induced IL-10 production in B cells and macrophages [19] and is involved in the control of the innate immune response suppressing the expression of TLR-mediated genes, including IL-6, in LPS-stimulated cells [20]. In murine uterine tissues, IL-6 downregulation correlates with IκBNS overexpression during certain phases of the reproductive cycle [21]. In the present work, we analyzed the behavior of IκBNS and its relationship with IL-6 expression within the development of several stages of healthy and infected pregnancy in a mouse model. Additionally, we also examined whether embryo implantation and fetal growth were affected by infection-related IL-6 and IκBNS expression.

2. Materials and methods

2.1. Ethical approval

All procedures involving animals were conducted under the ethical standards of the Escuela Nacional de Ciencias Biológicas-IPN. All applicable international, national, and institutional guidelines for the care and use of animals were followed. The registration and approval of the protocol is under the file number ENCB/CEI/016/2020, CONBIOETICA-09-CEI-002-20190327.

2.2. Mice and mating

BALB/c female (8–10 weeks age) and C57BL/6 male mice from Harlan Mexico Laboratories were maintained in controlled conditions of temperature (28 °C) and light/dark intervals (12 h). Rodent chow and plain water were provided *ad libitum*. All animal experiments were performed according to the appropriate guidelines for animal use approved by the Institutional Bioethics Committee of the National School of Biological Sciences. Cytology from fresh vaginal smears was observed under a light microscope to determine diestrous or estrous phase. Female mice at estrous phase were mated with males all night. The next morning, mating was confirmed upon detection of a vaginal plug and time of pregnancy was established as 0.5- days *post-coitum* (dpc). Uterine tissues from pregnant healthy mice (n = 4 per group) were recovered at 4.5, 5.5, 7.5, 10.5, 12.5, 18.5 dpc, labor (occurring at 20 dpc), 2- and 5-days *post-partum* (dpp).

2.3. Infection with Listeria monocytogenes

To confirm the infectiveness of bacteria, a suspension of 10^9 CFU/mL *L. monocytogenes* was prepared in sterile PBS and female BALB/c mice were inoculated i.v. with 100 μL of the bacterial suspension. To confirm infection, three days after inoculation CFU/mL was determined in harvested uteri, using PCR coupled to DNA sequencing of the 16S ribosome (data not shown). So then, new females were infected at diestrous and later on mated with healthy males. The uteri from these mice (n = 4 per group) were obtained at diestrous, 4.5, 5.5, 7.5, and 10.5 dpc. As a second group of infection, we worked with a different set of mice which was first mated and later on infected at 10.5 dpc to obtain the uteri at 18.5 dpc.

2.4. Real-time PCR

Uteri were homogenized individually and total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Two μg of total RNA quantitated by NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) were reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Gene expression was analyzed using the following TaqMan probes: ikbns (AppBio #Mm00549082_M1), il-6 (AppBio #Mm00446190_M1 Mm00446190_M1), and rplp0 (AppBio #

Mm00725448_S1). PCR was carried out in Step One Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using FastStart Taq-Man Probe Master (Hoffmann-La Roche, Basel, Switzerland). PCR program consisted in denaturing at 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s and annealing/elongation at 60 °C for 1 min.

2.5. Western blot

Total fraction of proteins was extracted from uteri by adding 400 μL of RIPA buffer (Tris-HCl pH 7.6, NaCl 150 mM, EDTA 2 mM, Glycerol 10%, Triton-X100 1%, sodium deoxycholate 0.5% and sodium lauryl sulfate 0.2%). Phenylmethylsulfonyl fluoride (Sigma, Saint Louis, MO, USA) and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) were also added. Proteins were quantitated by Lowry method (Bio-Rad Laboratories, Hercules, CA, USA). Samples were boiled and diluted 1:5 in Laemmli buffer (Bio-Rad Laboratories) and 2-mercaptoethanol (Bio-Rad Laboratories). 50 μg of total protein per sample were loaded on NuPAGE BIS-TRIS 10% gels (Invitrogen) and transferred to nitrocellulose sheets (Amersham Biosciences, Buckinghamshire, UK). Nonspecific binding was blocked with 5% non-fat dry milk in TBS-Tween 20 (0.5%) for 1 h at room temperature. Primary antibodies: monoclonal rabbit anti–IL-6 (1:1,000) D5W4V clone (Cell Signaling Technology, Danvers, MA, USA); polyclonal rabbit anti–NFKBID (IκBNS) (1:500) ab182633 (Abcam, Cambridge, MA, USA). Secondary antibody: goat anti-rabbit IgG–Peroxidase (1:5000) ab6721 (Abcam, Cambridge, MA, USA). Chemiluminescence was developed with Pierce ECL Western blotting substrate and detected on blue autoradiography film (Kodak, Rochester, NY).

2.6. Statistics

Results were expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA). Differences between group samples were evaluated with the non-parametric Mann-Whitney test.

3. Results and discussion

3.1. Physiological IκBNS low expression is reversed in an infection condition during implantation/placentation stage

Atypical nuclear IκB regulators play an important role in physiological regulated expression of pro-inflammatory cytokines, which is necessary during certain pregnancy stages such as embryo implantation [22, 23]. In this work, we analyzed the nuclear regulator IκBNS, along with IL-6, in healthy and *L. monocytogenes* – infected mice during implantation (4.5 dpc) and placentation (5.5, 7.5 and 10.5 dpc) and compared it to the expression in diestrous. We observed that at 4.5 dpc as well as 5.5 and 7.5 dpc the transcription of IκBNS showed no significant change and only a clear increase of 3-fold in transcription of IκBNS was found in placentation at 10.5 dpc (Figure 1A), while protein production showed a progressive increase starting at placentation (Figure 1B). Consistently, increased IL-6 transcription and protein production (Figures 1A and 1B) were observed during the whole implantation/placentation stage with a peak at 10.5 dpc which showed a 22-fold increase. IL-6 secreted from fetal-maternal tissues contributes in the balance of invasive properties of trophoblast cells through induction of integrins and matrix metalloproteases (MMPs), but it also possesses chemo-attracting activity to recruit proper immune cell types to ensure a proinflammatory environment. This cytokine participates as well in placental morphogenesis during and after implantation period exerting vascular remodeling through the induction of VEGF expression in uterine endothelial cells [24]; therefore, high levels of IL-6 are required during implantation and placentation. Furthermore, in an abortion-prone mouse model, placental tissues secrete low levels of IL-6 at 10.5 dpc [25], same time when we found the highest expression of this cytokine. It is clear
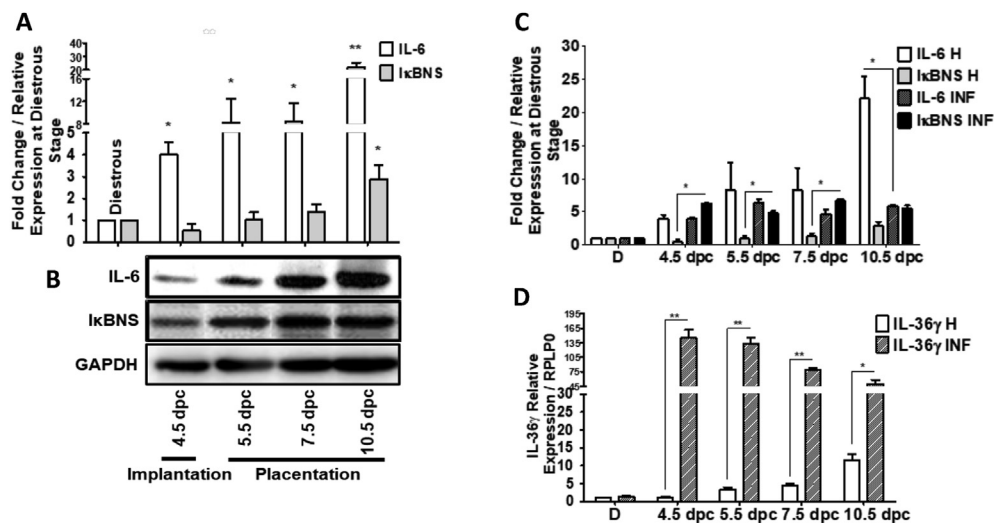
Figure 1. Expression pattern of IκBNS, IL-6 and IL-36γ in early pregnancy in healthy or infected conditions. Healthy mice were euthanized at diestrous, 4.5, 5.5, 7.5 and 10.5 dpc and uteri were recovered (n = 4) in order to assess IL-6 and IκBNS (A) mRNA expression relative to mice at diestrous and (B) protein production (original dots on supplementary figures 1B-1, 1B-2 and 1B-3). Additionally, (C) IL-6 and IκBNS mRNA expression in infected pregnant mice relative to infected mice at diestrous was analyzed and compared to healthy mice. (D) mRNA expression of inflammatory cytokine IL-36γ was also analyzed. Data normalized to housekeeping gene RPLP0; GAPDH protein expression as loading control. Results are shown as mean ± SEM. Mann-Whitney test *p ≤ 0.05; **p ≤ 0.005. *D*: diestrous; *dpc*: days post-coitum; *H*: healthy; *INF*: infected.

that IL-6 has a role in the healthy implantation and placentation processes, and accordingly to the antagonistic relationship with IκBNS, previously reported in uterine tissue during estrous cycle [21], we found that the regulator expression remained low except at 10.5 dpc. This result suggests that IκBNS intensification sets up in late placentation to negatively regulate IL-6, since later in pregnancy when the placentation process has completed and fetal development begins, IL-6 overexpression is no longer required. Due to the relevance of the G+ bacteria *L. monocytogenes* as a risk factor in pregnancy [26], we also evaluated the expression of IκBNS and IL-6 in pregnant mice with listeriosis. The main source of infection with this pathogen is contaminated food, but vertical transmission from mother to fetus, although rare, poses detrimental, even fatal consequences for pregnancy outcome and newborn welfare [27]. To analyze the impact of an infection on the expression of IκBNS and IL-6 during implantation/placentation stages of pregnancy, in the present study we inoculated female mice with *L. monocytogenes* at diestrous phase to promote a pre-implantation infection and uteri were later recovered at 4.5, 5.5, 7.5 and 10.5 dpc and normalized to infected condition at diestrous. It is known that several stimuli induce IκBNS expression, and accordingly, we noticed that IκBNS transcription increased in infected compared to healthy mice (Figure 1C) During a healthy pregnancy a combination of macrophages, DCs, uterine NKs and T cells amongst others immune cells, mostly in a tolerogenic state, is established in uterus to ensure a positive outcome [28] but in the case of an infection, macrophages and T cells change their phenotype. Activated macrophages show an increased IκBNS expression and repress IL-6 promoting cell survival [29]. Furthermore, it is known that TCR activation in lymphocytes induces IκBNS overexpression favoring the production of IL-2 and promoting cell proliferation [30]. In line with this previously described data, we observed that IκBNS expression was higher in uteri from infected compared to healthy mice, during most of the implantation/placentation period, except at 10.5 dpc when IκBNS transcription levels of the healthy group were similar to those of the infected counterpart. In contrast to the observations previously made in the healthy condition, IL-6 expression did not increase in the infection condition, including 10.5 dpc (Figure 1C). Di Simone *et al.* have also reported that there is no difference in IL-6 production in mice whether untreated or LPS-administered, from the beginning of pregnancy until 15 dpc [31]. Even though these results are similar to ours, in the infected condition we observed that IκBNS transcription is increased, opposite to the healthy pregnancy. Due to the absence of IL-6 overexpression found in infection condition, we evaluated IL-36γ transcription as well. IL-36γ is another pro-inflammatory cytokine which in contrast to IL-6, is not regulated by IκBNS. As previously reported, and as a marker of an inflammatory process in uterine tissue, IL-36γ was highly expressed during the

implantation/placentation period in infected mice compared to the healthy group (Figure 1D). This result indirectly shows that IL-6 induction is being particularly halted by an infection-induced IκBNS overexpression during an early stage of pregnancy. Given the relevance of IL-6 in the placentation process and its stage-specific expression pattern, deficiency of this cytokine is associated with impairment in reproductive function, causing fetal resorption for instance, as reported by Robertson *et al* [32] and in line with our results shown afterwards. On the other hand, higher bioavailability of IL-6 has been associated to elevated blood pressure, increased urinary protein excretion and decreased litter size and weight in pregnant rats [33], signs that resemble pre-eclampsia complications in women. Furthermore, Irvin *et al.* reported that pregnant guinea pigs infected with *L. monocytogenes* show a deficient systemic production of TNF-α [34], supporting the idea that not only IL-6, but other pro-inflammatory cytokines can be down-regulated in infection scenarios. Whether this effect depends on Bcl-3 (the nuclear regulator of TNF-α), similar to the IκBNS – IL-6 relationship, is still unknown.

3.2. IκBNS expression remains unchanged during fetal development stage but is overexpressed after delivery

Once the implantation stage has completed, pro-inflammatory cytokine expression is no longer necessary, therefore IL-6 presence must be down-regulated afterwards during the fetal development stage but is once again required for adequate delivery of the product. We also assessed the expression of IκBNS and IL-6 in healthy conditions at 12.5 dpc, 18.5 dpc, labor, and 2 and 5 dpp in comparison to the diestrous phase. We observed that IL-6 transcription (Figure 2A) and protein production (Figure 2B) dropped at 12.5 and 18.5 dpc with regard to implantation/placentation stage time points. Unexpectedly, IκBNS showed no difference during fetal development in contrast with previous observations in estrous cycle, where IL-6 reduction correlated with IκBNS overexpression. This led us to think that IL-6 and IκBNS behavior could be disrupted in the uterine tissue during healthy pregnancy. In our study, IL-6 mRNA and protein expression sharply increased during labor, without any change in IκBNS levels. Consistently with our data, Robertson *et al.* described that IL-6 KO mice deliver their pups 24 h later than WT control mice [35]. IL-6 overexpression found in labor was followed by a constriction at 2 and 5 dpp, whereas IκBNS transcription and protein expression had higher levels at 2 dpp (Figure 2A). In mice, postpartum estrus may occur, which is ovulation and corpus luteum production 14–24 h following parturition [36], which in our case could explain the results observed after delivery, besides IκBNS increase may have place at this time point to regulate estrous cycle once again. IκBNS is known for its suppressive activity on certain pro-inflammatory NF-κB target genes. For
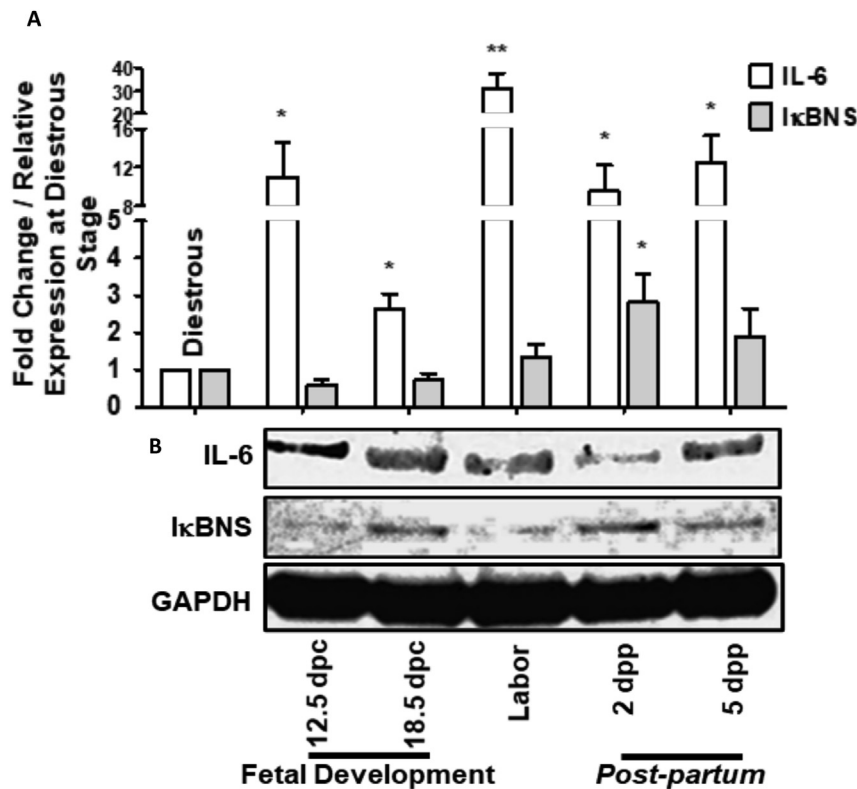
A

Figure 2. Expression pattern of IkBNS and IL-6 in late pregnancy and *post-partum* in healthy conditions. Healthy mice were euthanized at diestrous, fetal development, labor and post-partum stages and uteri were recovered (n = 4) in order to assess IL-6 and IkBNS (A) mRNA expression relative to mice at diestrous and (B) protein production in a normal pregnancy condition (Original dots on supplementary figures 2B1 and 2B2). Data normalized to housekeeping gene RPLP0; GAPDH protein expression as loading control. Results are shown as mean ± SEM. Mann-Whitney test *p ≤ 0.05; **p ≤ 0.005. *dpc*: days post-coitum; *dpp*: days post-partum.

instance, in cells stimulated with LPS, late stages of IL-6 transcription are regulated by IκBNS, which favors p50 homodimer binding to DNA in the promoter region of IL-6 gene to repress its transcription [20]. In spite of observed IL-6 levels that promote both correct fetal development and labor, IκBNS behavior did not turn out as expected. The reduced amount of animals per group employed in the assays may represent a limitation for plausible explanations to this result; although the use of syngeneic mice strains under tightly controlled conditions allows us to suggest that IL-6 regulation, at least in a healthy pregnancy, could be accounted for another atypical IκB regulator such as IκBζ, given that LPS-induced production of IL-6 is severely impaired in macrophages from IκBζ K.O. mice [37]. It is to note that in many cases, IκBNS negative regulation over IL-6 has been reported in cells (particularly macrophages) stimulated *in vitro* with pathogen associated molecular patterns or with pathogenic agents, but in the case of a healthy pregnancy there is no such stimulus, IκBNS expression remains low as before mentioned. In consequence, we sought to analyze the effects of post-implantation infection condition on the IκBNS/IL-6 expression.

3.3. IκBNS downregulation correlates with IL-6 overexpression in post-implantation infection

Several reports indicate that IL-6 is over-expressed in amniotic fluid, placenta and myometrium in PTL caused by infection. Besides testing a pre-implantation infection condition, we also inoculated mice with *L. monocytogenes* at 10.5 dpc and later retrieved the uteri solely at 18.5 dpc and named this condition post-implantation infection. In this case, IκBNS expression levels were sharply reduced at 18.5 dpc in infected mice compared to the healthy counterpart, whereas IL-6 expression was increased (Figure 3A), confirming even further the inverse relationship between these two molecules, and contrasting with the effect observed in the healthy pregnancy condition. IL-6 is an important cytokine in the generation of CD4+ Treg cells necessary for a successful pregnancy and it has been described that high levels of systemic IL-6 are related to recurrent miscarriage in fertile women [24]. Additionally, high levels of IL-6 in fetal -maternal membranes are found in abortion-prone mice at
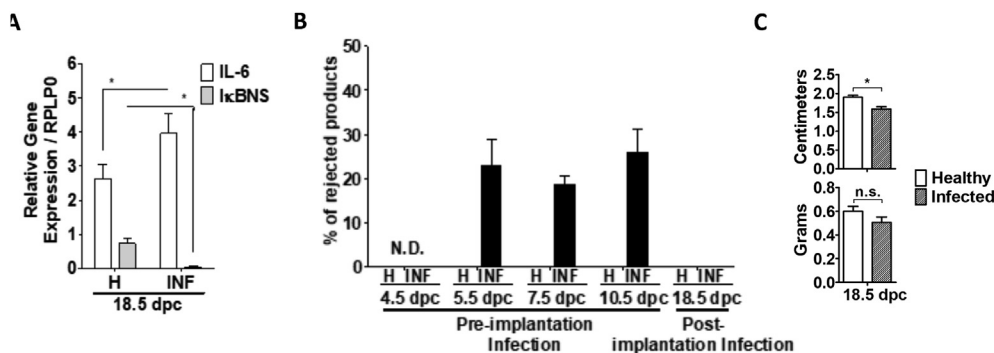

Figure 3. IkBNS downregulation correlates with IL-6 overexpression and decrease of fetal growth in post-implantation infection, while pre-implantation infection correlates with product rejection. Mice were infected with *L.monocytogenes* at diestrous or at 10.5 dpc and uteri were retrieved at 4.5 dpc, 5.5 dpc, 7.5 dpc, 10.5 dpc and 18.5 dpc respectively (n = 4). (A) IL-6 and IkBNS relative mRNA expression and (C) size and weight of fetuses was analyzed and compared to the healthy counterpart. (B) Percentage of rejected products for each litter was recorded in infected mice compared to healthy mice. Results are shown as mean ± SEM. Mann-Whitney test *p ≤ 0.05. *N.D.* not determined; *n.s.* not significant. *dpc*: days post-coitum; *H*: healthy; *INF*: infected.

day 18 of pregnancy [38]. Therefore, we investigated whether the observed results correlate to embryo viability and fetal development.

3.4. Embryo viability and fetal growth are affected in the infection condition

We examined the number of rejected or unviable products per litter in pre-implantation infection, as well as the average size and weight of fetuses in post-implantation infection compared to healthy conditions. At 4.5 dpc implantation sites were visible but embryos were barely identifiable, so distinguishing a viable product from a rejected one was not possible. It is to note that pre-implantation infection, which showed less expression of IL-6, promoted the rejection on average of at least 20% of the total products in each litter, compared to healthy conditions where all products were viable (Figure 3B). It remains unknown whether this consequence is attributable to IL-6 low expression alone or to the presence of *L. monocytogenes* in uterine tissues, but additional variables for instance the exacerbated expression of IL-36γ may take part in this effect. Lack of IL-6 overexpression in infected mice could be the cause of product rejection, but also the increase in IκBNS may be involved. Besides playing a role in TLR-mediated gene repression, IκBNS could also indirectly impact on endometrial vascular remodeling required during the implantation/placentation stage. For instance, Niida *et al.* reported that vascular injury performed in IκBNS K.O. mice caused an increase in the area of the intima from femoral artery blood vessel, accompanied with elevated transcription of IL-6, compared to WT mice [39]. In our study we observed the opposite effect, since the pre-implantation infection caused IκBNS overexpression and limited IL-6 induction. This in consequence could also affect placentation development in certain implantation sites due to a disruption in vascular remodeling. Although IL-6 null mutant mice show frequent fetal resorption, other processes such as estrous cycle, ovulation, fertilization and embryo implantation are not affected in these animals. Moreover, reduced IL-6 protein and mRNA expression has been observed in endometrial tissues from women prone to recurrent spontaneous abortion [24]. Unlike other reports [38], we found that post-implantation infection (which caused a reduction in IκBNS and an increase in IL-6 expression) had no impact on embryo viability (Figure 3B). If infection occurs after the completion of embryo implantation, IκBNS expression drops allowing for IL-6 induction to promote an inflammatory response that may counter the infection source, but not that high to increase the rate of abortion. On the other hand, we observed that post-implantation infection significantly reduced product size without affecting their weight (Figure 3C), meaning that even though the products are not rejected, they are affected by the presence of pro-inflammatory cytokines, IL-36γ included.

4. Conclusions

Altogether, these results suggest that IκBNS could be expendable for IL-6 regulation during the development of a healthy pregnancy, but in the case of a pregnancy preceded by an infection, IκBNS seems to restrict IL-6 overexpression and embryo arrestment occurs. It remains unknown whether this consequence is attributable to IL-6 low expression or to the presence of *L. monocytogenes* in uterine tissues, but additional variables for instance the exacerbated expression of IL-36γ, even the high expression of IκBNS may take part in this effect. As for the case of a mid-pregnancy infection, fetal growth restriction, without product abortion, can also be a consequence of IL-6 overexpression promoted by IκBNS low expression.

Declarations

Author contribution statement

S. Rodríguez: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

M. Cancino-Díaz: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

J. Murrieta-Coxca and D. Baeza-Martínez: Performed the experiments.

Ó.H. López-Portales and F. Gómez-Chavez: Analyzed and interpreted the data; Wrote the paper.

J. Cancino-Díaz: Analyzed and interpreted the data.

S. Pérez-Tapia: Contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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